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HPV-16 L1 VLP vaccine elicits a broad-spectrum of cytokine responses in whole blood

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Abstract

Here, we evaluated innate and adaptive immune system cytokine responses induced by HPV-16 L1 VLP in whole blood (WB) cultures from individuals receiving the vaccine ($n = 20$) or placebo ($n = 4$) before and after vaccination. 11 cytokines were measured: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α, and GM-CSF using multiplex bead arrays. Cytokine profiles from WB samples clearly discriminated between vaccine and placebo recipients and between pre and post-vaccination responses. Significant increases in Th1, Th2 and inflammatory cytokines were observed in WB assays following vaccination. Results from WB assays were compared against parallel PBMC-based assays in a subset of patients. Differences between whole blood assay and PBMC were observed, with the highest levels of induction found for WB for several cytokines. Our results indicate that multiplex assays for cytokine profiling in WB are an efficient tool for assessing broad spectrum, innate and adaptive immune responses to vaccines and identifying immunologic correlates of protection in efficacy studies.

Keywords: HPV-16 L1 VLP vaccine; Cytokines; Whole blood

1. Introduction

A vaccine that prevents persistent HPV-16 infection could substantially reduce the incidence of cervical cancer and its precursors. One candidate prophylactic HPV vaccine utilizes virus-like particles (VLPs), which are empty non-infectious viral capsids that structurally mimic the outer shell of the virion [1–4]. In animal papillomavirus models, systemic immunization with L1 VLPs can induce high titers of neutralizing antibodies that confer protection against high-dose experimental papillomavirus challenge [5,6]. Intramuscular vaccination with L1 VLPs in humans has been well tolerated and induced high serum antibody titers (40-fold higher than titers seen following natural infection) as well as cell mediated immune responses, including T cell proliferative (CD4 and CD8) and cytokine responses [7–10]. Furthermore, a recent proof of principle HPV16 L1 VLP efficacy trial has shown excellent protection against persistent HPV16 infection suggesting that immunization with HPV 16 L1 VLP may reduce the risk of cervical cancer [11]. Protection is likely conferred by the generation of high levels of neutralizing antibodies. However, the possible role of T-cell responses and the involvement of T-cells in mediating production of neutralizing antibodies and antiviral effect are poorly understood. Cytokine profiling, as a measure of T cell responses, may offer insights into mechanisms of protection against disease and help explain the consistently strong antibody responses

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induced by the vaccine. We and others have found that vaccination with L1 VLP in humans was associated with increases in ex-vivo production of Th1 (IFN-γ) and Th2 type cytokines (IL-5, IL-10) by peripheral blood leukocytes stimulated in vitro with the vaccine [8–10]. However, the complete spectrum of cell-mediated responses has not been characterized.

Whole blood assays provide a simple tool for assessing immune cytokine profiles. These assays have previously been used to assess the immune status of individuals with various conditions [12–18]. These studies have used conventional techniques of cytokine determination, such as enzyme-linked immunosorbent assays (ELISA), which generally require large quantities of cells and supernatant to characterize cytokine profiles. Recently, new fluorescent-bead-based techniques have been developed to allow the measurement of multiple cytokines in a single assay using limited amounts of material. The multiplex cytokine assays provide a unique system for the investigation of the roles of different cytokines in various pathologic states [19–24].

Whole blood cytokine induction assays combined with the multiplex cytokine detection technology allow evaluation of innate and adaptive cytokine responses in a single assay, through quantitation of inflammatory, Th1 and Th2 type cytokines. These assays have not been previously used for monitoring of cytokine responses to vaccines but they have potential for use in the field as immune monitoring assays, since they require small volumes of blood, basic laboratory facilities, are characterized by few preparation artifacts and, deliver standardized performance. This might be of special interest for large-scale immuno-epidemiological vaccine studies in which cell isolation may be difficult to accomplish.

The goals of the current study were: (1) to better characterize the innate and acquired immune system cytokine responses elicited by L1 VLP vaccination; (2) to assess whether an ex-vivo whole blood cytokine induction assay would allow identification of vaccine responders by discrimination of cytokine profiles between placebo and vaccine recipients before and after vaccination; and (3) to compare cytokine responses to L1 VLP in whole blood with responses in PBMC.

2. Materials and methods

2.1. Study design

A double-blind, randomized, placebo-controlled trial of the L1 HPV-16 VLP vaccine without adjuvant was conducted, in 24 healthy, HIV-seronegative adult female volunteers 18–25 years of age. This group of 24 women consisted of a subset of participants in a larger program designed to examine the safety and immunogenicity of the L1 HPV-16 VLP vaccine. Subjects determined by sexual history to be at low risk for HPV16 exposure were enrolled at The John Hopkins University Center for Immunization Research (Baltimore, MD). Based on previous results [7], a 50 μg dose without adjuvant was used. Women were randomly assigned to one of two groups to receive 50 μg L1 HPV-16 vaccine ($n = 20$) or placebo ($n = 4$), 0.5 ml of sterile saline solution. Subjects received vaccine or placebo intramuscularly into the deltoid at 0, 1, and 6 months. Subjects were evaluated clinically and blood specimens were collected for immunologic assays prior to the initial vaccination (month 0) and 1 month following each boost vaccination (months 2 and 7). All vaccine recipients were monitored for clinical signs and symptoms for 7 days after each vaccination. The vaccine was well tolerated and induced high levels of antibodies, as reported in the previous trial [7]. The protocol for this study was approved by the The John Hopkins University Institutional Review Board. The blood specimens earmarked for cytokine assays were shipped fresh to the monitoring laboratory for processing and testing.

2.2. HPV-16 L1 VLP vaccine

Recombinant human papillomavirus (HPV) type 16 L1 virus-like particles (VLPs) expressed in the baculovirus system were used to investigate the cellular immune response to VLP vaccination. HPV-16 L1 VLPs were expressed in baculovirus-infected Sf9 insect cells (Novavax, Rockville, MD). Production of clinical lots of recombinant HPV-16 L1 VLP vaccines was performed in accordance with GMP guidelines at the vaccine production facility of Novavax, Inc., as previously reported [7]. Formulated VLPs from a single lot were dispensed aseptically into sterile vials (3.0-ml size, type 1 borosilicate glass, silanized, depyrogenated; Wheaton Glass, Wheaton, MD) as a single-unit dose and were designated final container vials. Vials containing VLP antigen were stored at −80 °C and thawed immediately preceding administration.

2.3. Whole blood cytokine induction assay

Heparinized blood was collected from 20 vaccine and 4 placebo recipients at month 0, 2, and 7. Freshly collected blood (within 4 h) was cultured undiluted (1 ml) in the presence of media alone (control cultures), HPV-16 L1 VLP (10 and 1 μg/ml, Novavax) and phytohaemagglutinin (PHA 2 μg/ml, Sigma, St. Louis, MO, USA), for 24 h at 37 °C in a 5% CO_2-incubator in tissue culture tubes. Cells were then removed by centrifugation and supernatants were collected and stored at −20 °C until cytokine testing was performed.

2.4. Mononuclear cells cytokine induction assay

PBMC were isolated from heparinized whole blood by density gradient centrifugation [25]. Isolated PBMC were cryopreserved for future testing in RPMI-1640 (Gibco, Invitrogen Life Technologies, Carlsbad, CA) supplemented with penicillin-streptomycin (100 μg/ml–100 U/ml, Gibco), Glutamine (2 mM), HEPES buffer (10 mM), 20% FCS (Hyclone) and 7.5% DMSO (Sigma). Cryopreserved PBMC from six vaccine recipients for which cells were available and

for whom whole blood cultures had also been set up were thawed and utilized for cytokine induction assays. PBMC (at a final concentration of 1.5×10^6/ml) were incubated in the absence or presence of PHA (2 μg/ml), Influenza virus (Flu, 1:100), L1 HPV-16 VLP (10, 2.5, and 1 μg/ml) for 3 days at 37 °C and 6% CO_2 in RPMI-1640 supplemented with penicillin/streptomycin (100 μg/ml/100 U/ml), Glutamine (2 mM) and HEPES buffer (10 mM) and 10% FCS. Cell free supernatants were harvested and frozen at −20 °C. A Sf-9/baculovirus insect cell lysate (0.1 μg/ml, Novavax) was used as a control antigen in the PBMC cytokine induction assays. Stocks of VLP preparations were provided by the vaccine manufacturer at a concentration of 1.0 mg/ml. The purity of the HPV-16 VLPs was >96% as determined by SDS-PAGE.

2.5. Cytokine determinations

Supernatants from the whole blood and PBMC cytokine induction assays were thawed and tested in duplicate wells for the following 11 cytokines: IL-1-β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α, and GM-CSF, using multiplex kits (Linco Research, St. Louis, MO) following manufacturers' instructions. In brief, the assay is based on conventional sandwich assay technology. The antibody specific to each cytokine is covalently coupled to Luminex microspheres, with each antibody coupled to a different microsphere uniquely labeled with a fluorescent dye. The microspheres are incubated with standards, controls, and samples (25 μl) in a 96-well microtiter filter plate for 1 h at room temperature. The plate is then washed to remove excess reagents, and biotin-labeled detection antibody, in the form of a mixture containing each of the eleven antibodies, is added. After a 30-min incubation at room temperature, streptavidin–phycoerythrin conjugate is added for an additional 30 min. After a final wash step, the beads are resuspended in buffer and read on the Bioplex Instrument (Biorad, Hercules, CA) to determine the concentration of the cytokines of interest. All specimens were tested in replicate wells. Results were reported as the mean of the replicates. Data analysis was performed using Bioplex Manager software v 2.0. A five parameter logistic curve fit was applied to each standard curve and sample concentrations were interpolated from the standard curve. The lower levels of detection for all the cytokines were 3.2 pg/ml. In this analysis, specimens with individual cytokine levels below the lower detection limit were arbitrarily assigned a value of 1/2 of this value (1.6 pg/ml, respectively) for that cytokine. A plasma/serum matrix supplied by Linco was used when plasma samples were tested. To simplify presentation, cytokines were grouped into three categories: Th1 type (IL-2, TNF-α, IFN-γ, GM-CSF), Th2 type (IL-4, IL-5, IL-6, IL-10) or inflammatory (IL-1β, IL-8).

2.6. HPV-16 serology

Presence of serum IgG specific antibodies against HPV-16 VLPs was assessed by enzyme-linked immunosorbent assays (ELISA) as previously described [7]. Four-fold dilutions of each serum were assayed starting at dilution of 10. The antibody titers were given as the reciprocals of the highest dilution showing positive reactivity in each assay. Sera were designated ELISA positive at a given dilution if the absolute optical density was greater than or equal to 0.2 and was at least double the reactivity of the same serum dilution in a well containing blocking buffer but no VLP. Median antibody titers prior to vaccination in vaccine recipients ($n = 20$) were 0 (range 0–160). Following vaccination, median antibody levels were 2560 at months 2 and 7 (with ranges of 640–10240 and 640–20480, respectively). Only one of the 20 vaccine recipients was seropositive at entry with an antibody titer of 160. Median antibody titers in placebo recipients at enrolment were 0 (range 0–640). At months 2 and 7, median antibody titers were 0 (range 0–640 and 0–160, respectively).

2.7. Statistical analysis

The nonparametric Kruskal–Wallis or Mann–Whitney test was used to determine statistical differences for in vitro cytokine production between different culturing conditions for the same cellular preparation (PBMCs or whole blood) and between PBMCs versus whole blood for the same culturing conditions for a given time point. A nonparametric test for trend was used to test for time trends for in vitro cytokine production by either PBMCs or whole blood within a treatment group (vaccine or placebo) for a given culturing condition. A nonparametric test for trend was also used to test for trends in cytokine responses to differing amounts of VLP (0, 1, and 10 μg) for a given cell preparation at a given time point. To determine the relationships between different immune markers, we used Spearman rank correlation for continuous values. A p value <0.05 was considered significant.

3. Results

3.1. Cytokine production in response to L1 VLP in whole blood following vaccination

The cytokine profiles of in vitro activated whole blood from HPV-16 L1 VLP vaccine ($n = 20$) and placebo ($n = 4$) recipients were measured. Table 1 presents the median and mean cytokine levels in pg/ml in whole blood supernatants from unstimulated (media) cultures and cultures stimulated with L1 VLP (at 10 and 1.0 μg/ml) or a positive control (PHA) tested at month 0, 2 and 7 (before first immunization and 1 month following second and third immunizations). Fig. 1 (panels A–D) compares the responses of whole blood cultures from vaccine and placebo recipients to these different stimuli. Vaccination with L1 VLP induced an overall increase in cytokine production by whole blood in response to L1 VLP. Stimulation of cells from vaccine recipients with L1 VLP (10 μg/ml) induced significant increases in the median levels of inflammatory (IL-1β, IL-8), as well as Th1 (IL-2 and

Table 1
Cytokine production by whole blood cultures stimulated with L1 VLP (10 and 1 μg/ml) PHA and media control in vaccine and placebo recipients

Cytokine	Time (month)[a]	Media						L1 10 μg						L1 1 μg						PHA					
		Vaccine			Placebo			Vaccine			Placebo			Vaccine			Placebo			Vaccine			Placebo		
		Median	Mean	*p*	Median	Mean	*p*	Median	Mean	*p*	Median	Mean	*p*	Median	Mean	*p*	Median	Mean	*p*	Median	Mean	*p*	Median	Mean	*p*
IL-2	0	1.6	1.8		1.6	1.6		2.5	7.0		2.6	3.4		1.6	3.2		1.6	2.7		371.9	415.2		556.6	607.4	
	2	1.6	1.7		1.6	1.6		117.3	121.3		1.6	2.3		16.4	18.9		1.6	1.6		444.5	429.9		734.1	702.8	
	7	1.6	1.9	NS[b]	1.6	1.6	NS	130.0	178.3	<0.001	2.6	2.9	NS	16.0	32.6	<0.001	1.6	2.7	NS	309.0	369.7	NS	987.7	952.6	NS
IFN-γ	0	2.5	4.4		1.6	4.9		3.6	5.2		3.7	5.8		3.7	4.0		2.9	5.7		250.4	311.6		569.5	571.7	
	2	2.5	4.0		1.6	5.0		26.3	27.9		2.4	5.2		4.9	6.3		1.6	5.7		272.6	313.0		673.1	766.3	
	7	1.6	4.5	NS	1.6	5.6	NS	24.2	44.6	<0.001	1.6	4.9	NS	7.9	7.6	0.04	1.6	6.1	NS	211.5	257.5	NS	765.3	1118.7	NS
TNF-α	0	3.6	3.8		2.6	2.9		3.7	6.4		4.0	10.8		3.5	5.1		3.2	3.2		135.9	160.6		466.0	395.2	
	2	3.3	3.2		2.6	2.8		15.8	57.3		1.6	2.3		4.7	7.6		2.7	3.4		127.8	154.4		422.0	430.6	
	7	1.6	3.3	NS	3.5	3.2	NS	17.9	56.8	<0.001	2.7	2.9	NS	5.8	134.0	NS	3.9	3.5	NS	72.6	154.7	NS	603.0	586.5	NS
IL-12	0	1.6	2.7		1.6	2.0		1.6	2.7		1.6	1.6		1.6	2.6		1.6	2.2		1.6	3.1		3.4	3.0	
	2	1.6	2.5		1.6	1.6		1.6	2.5		1.6	2.0		1.6	2.6		1.6	2.1		1.6	2.8		2.8	3.1	
	7	1.6	2.7	NS	1.6	2.4	NS	1.6	2.4	NS	1.6	2.1	NS	1.6	2.7	NS	1.6	2.6	NS	1.6	2.8	NS	2.4	3.3	NS
GM-CSF	0	1.6	2.4		1.6	2.2		1.6	2.5		1.6	2.3		1.6	2.2		1.6	2.2		28.2	27.2		46.0	88.1	
	2	1.6	2.0		1.6	2.0		7.2	8.6		1.6	2.1		1.6	2.4		1.6	2.2		32.3	36.0		44.3	103.8	
	7	1.6	2.3	NS	1.6	1.6	NS	11.2	12.2	<0.001	1.6	1.6	NS	1.6	5.6	0.03	1.6	1.6	NS	26.7	30.4	NS	101.8	167.0	NS
IL-4	0	8.9	27.6		2.7	5.8		10.6	27.1		4.2	6.4		9.4	26.4		3.3	5.9		81.5	91.6		91.3	83.6	
	2	8.3	27.2		2.4	5.1		26.6	42.1		2.5	5.7		10.3	29.4		1.6	5.4		82.5	94.7		113.8	108.4	
	7	10.2	27.8	NS	2.8	5.0	NS	43.5	60.5	0.002	3.2	5.0	NS	21.6	33.8	NS	2.6	5.0	NS	88.5	91.2	NS	170.4	170.6	NS
IL-5	0	1.6	2.0		1.6	1.6		1.6	2.1		1.6	1.6		1.6	2.0		1.6	1.6		3.8	4.6		1.6	3.3	
	2	1.6	2.1		1.6	1.6		1.6	3.1		1.6	1.6		1.6	1.7		1.6	1.6		4.3	5.3		1.6	4.1	
	7	1.6	2.0	NS	1.6	1.6	NS	1.6	6.1	0.007	1.6	1.6	NS	1.6	2.8	NS	1.6	1.6	NS	1.6	4.8	NS	5.7	15.9	NS
IL-6	0	25.9	36.4		21.5	17.5		44.9	134.0		30.9	111.9		36.9	67.9		21.9	23.0		851.8	1223.0		2184.7	2564.1	
	2	23.8	42.1		24.1	29.0		1135.7	2486.3		32.6	84.5		104.5	636.7		16.6	49.5		878.9	1439.9		2413.5	3173.4	
	7	21.4	128.8	NS	13.3	14.3	NS	512.0	1870.0	<0.001	11.9	14.6	NS	302.7	1342.8	<0.001	13.3	12.7	NS	654.9	1228.7	NS	2751.2	3176.8	NS
IL-10	0	1.6	4.3		1.6	2.0		3.8	5.1		2.7	2.9		2.6	4.3		2.7	3.0		213.0	234.7		149.6	405.4	
	2	1.6	3.2		1.6	2.2		16.7	23.5		1.6	2.3		6.1	10.0		2.6	2.8		172.5	223.1		269.3	336.9	
	7	1.6	4.6	NS	2.5	2.5	NS	24.1	50.5	<0.001	1.6	2.0	NS	14.6	24.7	<0.001	1.6	2.2	NS	181.2	303.3	NS	468.3	491.6	NS
IL-1β	0	4.0	3.5		4.3	3.6		3.7	4.8		5.7	5.7		3.5	3.4		4.5	4.7		30.4	44.3		201.1	221.6	
	2	3.6	3.9		2.6	2.7		20.1	45.8		4.3	4.6		6.5	10.2		3.6	3.3		31.8	59.4		187.9	185.2	
	7	2.5	4.4	NS	2.5	2.7	NS	12.6	39.9	0.001	2.5	2.7	NS	6.2	145.3	0.005	2.6	3.1	NS	25.6	61.9	NS	243.2	234.0	NS
IL-8	0	559.8	783.0		511.7	631.5		872.8	1775.4		734.7	1052.6		644.5	1097.7		796.0	897.8		4461.4	5295.2		6127.1	6787.9	
	2	555.8	953.7		560.6	549.1		8917.8	7410.4		623.1	876.5		2708.3	3826.8		455.4	679.0		5949.5	6084.2		8139.9	7756.8	
	7	463.1	1011.9	NS	347.2	418.0	NS	7579.3	7058.3	<0.001	556.8	649.1	NS	3769.0	4571.9	<0.001	464.8	528.3	NS	3799.3	5180.5	NS	7659.6	7675.7	NS

All cytokine levels expressed in pg/ml determined as indicated in Section 2. P_{trend}, a non-parametric test for trend was used to examine time trends in cytokine production for each culturing condition.

[a] Time post first immunization.

[b] NS = Non significant $p > 0.05$.

IFN-γ, TNF-α, GM-CSF) and Th2 type (IL-4, IL-6, and IL-10) cytokines (Table 1, Fig. 1A). These results suggest that a recall response to L1 VLP vaccine comprises activation of innate and adaptive immune responses. The highest increment in cytokine response for whole blood cultures from vaccinated women relative to month 0 (baseline) was observed following the injection of the second dose of vaccine at month 2 for all the cytokines measured. Further increases following the third vaccination were seen for IL-2, TNF-α, GM-CSF, IL-4, IL-10, and GM-CSF, but these increases were mostly small and non-significant (Table 1, Fig. 1A).

Overall, the greatest relative increases in cytokine responses in whole blood cultures following vaccination were seen for IL-2 (47 and 52-fold at months 2 and 7, respectively), IL-6 (26- and 11-fold at months 2 and 7), and IL-8 (10- and 9-fold at month 2 and month 7), followed by GM-CSF (five- and seven-fold at months 2 and 7), IFN-γ (seven-fold at months 2 and 7) and IL-10 (four- and six- fold at months 2 and 7), TNF-α (four- and five-fold at months 2 and 7), IL-1β (five and three-fold at months 2 and 7) and IL-4 (three- and four-fold at months 2 and 7). Some L1 VLP recipients showed small increases for IL-5 and IL-12 production (in 9/20 and 4/20 recipients, respectively) in response to L1 VLP following vaccination.

Similar patterns of cytokine production to the ones seen in response to L1 VLP at 10 μg/ml were observed when L1 VLP was tested at 1.0 μg/ml (Fig. 1B, Table 1), but the magnitude of increase of cytokine production following vaccination was much smaller, although still statistically significant for most cytokines, when compared to pre-vaccination levels. In contrast to the vaccination-specific increase in median GM-CSF production in response to the 10 μg/ml L1 VLP dose, no changes for median levels of this cytokine were observed in response to L1 VLP tested at 1.0 μg/ml. Also, increases that did not reach statistical significance were found for TNF-α and IL-4, when VLP was tested at 1 μg/ml.

No significant increases in median cytokine levels produced in response to L1 VLP stimulation were observed among the placebo recipients at months 2 and 7 relative to month 0 ($p > 0.05$) (Table 1, Fig. 1A–D). No statistically significant increases in cytokine responses to PHA (Fig. 1C),

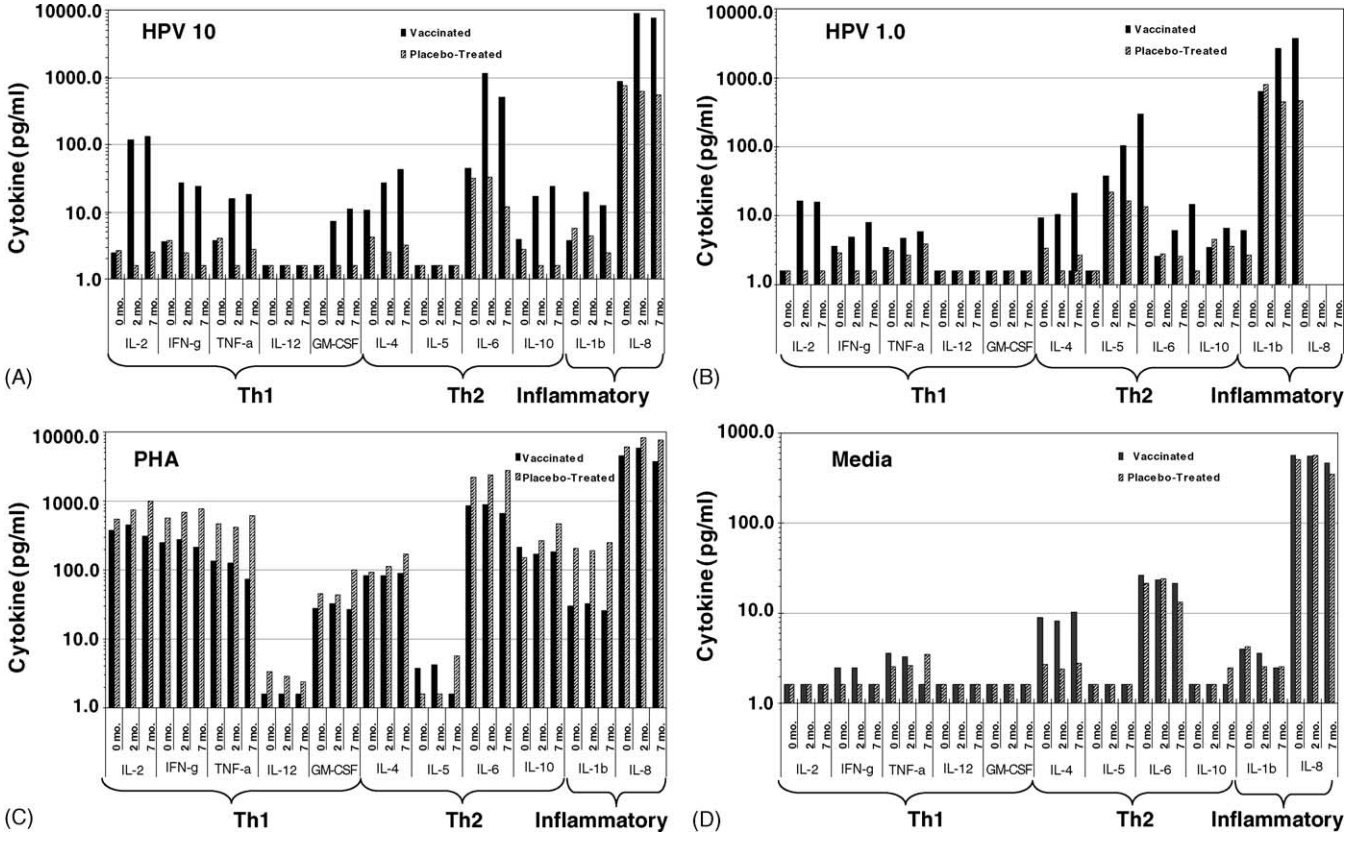

Fig. 1. Whole blood cytokine responses to HPV-16 L1 VLP tested in vitro either at 10 μg/ml (A) or 1 μg/ml (B), PHA (C), and unstimulated, media control (D) in a total of 20 vaccine (filled bars) and four placebo (hatched bars) recipients. Results are presented as median cytokine levels in pg/ml. Vaccine was administered at 0, 1, and 6 months. Whole blood collected at 0, 2, and 7 months (before and 1 month following second and third immunization) was stimulated for 24 h in the absence or presence of L1 VLP or PHA. Supernatants were collected and tested for cytokine content using multiplex cytokine analysis, as described in Section 2. Cytokines were divided in three categories (Th1, Th2 type and inflammatory) for simplicity of presentation. A non-parametric test of trend was used to examine time trends in cytokine production for each in vitro culturing condition.

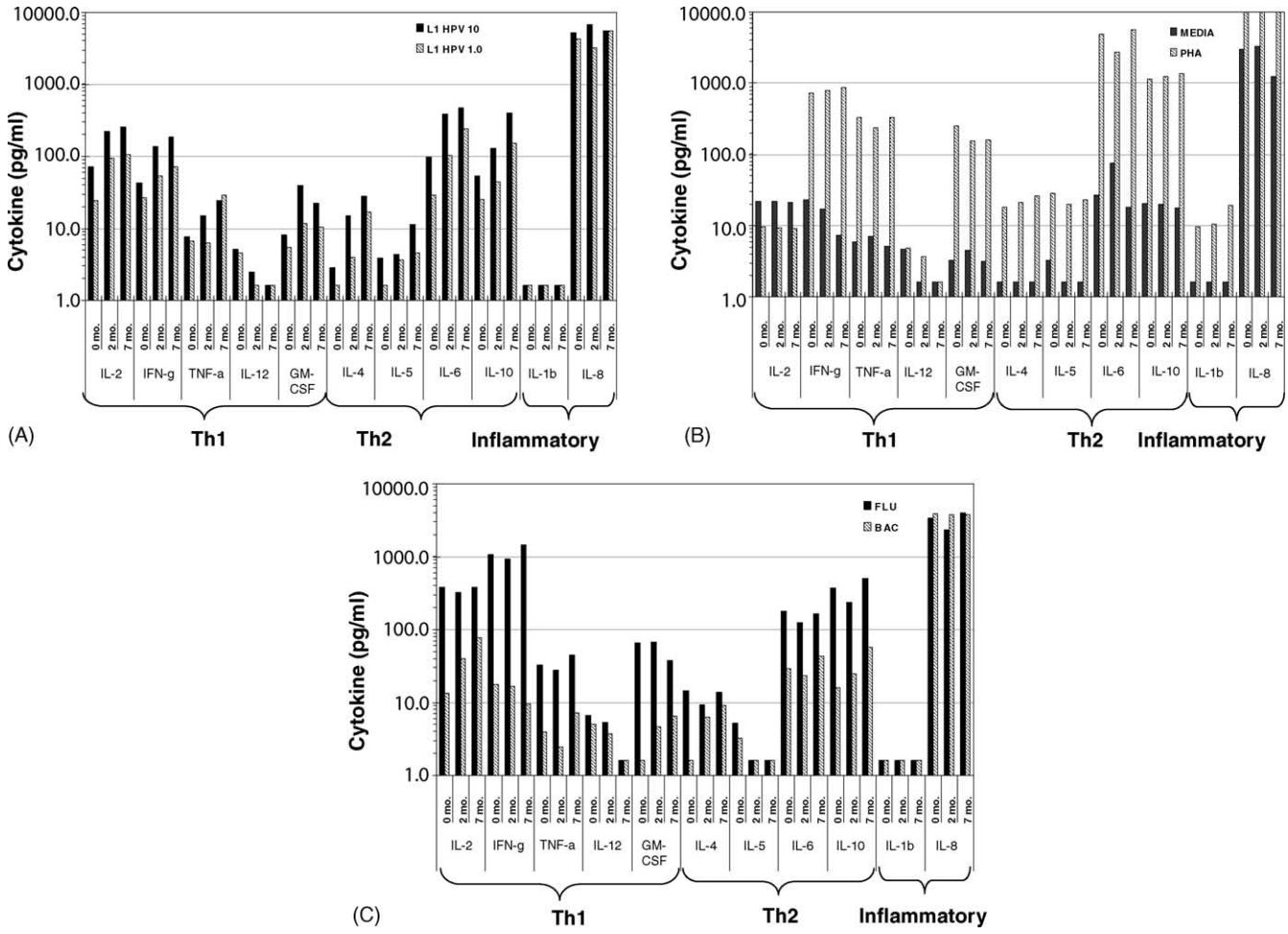

Fig. 2. PBMC cytokine responses to HPV-16 L1 VLP at 10 or 1 μg/ml (A), media control (unstimulated cultures) or PHA stimulated cultures (B), and influenza virus or baculovirus lysate (C) in six vaccine recipients. PBMC were stimulated for three days in vitro in the absence or presence of each of the stimuli as described in Section 2. Supernatants were collected and tested for cytokine content using multiplex cytokine analysis. Results are expressed as median cytokine levels (pg/ml).

in vaccine or placebo recipients following vaccination, were detected when compared to pre-vaccination responses.

As shown in Fig. 1D, spontaneous secretion of cytokines in the absence of any stimuli (media control) did not show any significant increases following vaccination. Cytokine levels in unstimulated cultures were negligible at month 0 in both vaccine and placebo recipients, with the exception of IL-4 (8.9 pg/ml for vaccine recipient), IL-6 (25.9 and 21.5 pg/ml for vaccine and placebo recipients) and IL-8 (559.8 and 511.7 pg/ml for vaccine and placebo recipients) (Table 1).

Stimulation of whole blood collected prior to vaccination ($n = 24$, vaccine and placebo groups combined) with increasing doses of L1 VLP (0, 1, and 10 μg/ml) resulted in increases that were not statistically significant in the production of IL-6 (median levels of 23.2, 32.7, and 39.8 pg/ml, respectively; $P_{\text{Trend}} = 0.07$) and IL-8 (median levels of 559.8, 644.5, and 817.8 pg/ml, respectively; $P_{\text{Trend}} = 0.05$). These results support previous studies that showed induction of inflammatory cytokine production by VLP, independently of vaccination [26–28].

3.2. Correlations between cytokines induced by L1 VLP following vaccination in whole blood

Analysis of correlations between cytokines produced in response to L1 VLP following vaccination at months 2 and 7 in vaccine recipients revealed poor correlation between the majority of the 11 markers evaluated (85 and 78% of the correlations at L1 VLP concentrations of 1 and 10 μg, respectively, ranging from −0.40 to 0.40). The largest (Spearman correlation > 0.60) and most significant correlations observed included the correlation between IL-10/TNF-α ($r = 0.78$, $p < 0.0001$) and IL-10/IL-6 ($r = 0.72$, $p < 0.0001$) when VLP was tested at 1 μg/ml. At 10 μg/ml, the largest (Spearman correlation > 0.60) and most significant correlation was found between IFNγ/IL-2 ($r = 0.63$, $p < 0.0001$). No significant correlations (ranging from −0.26 to 0.29) were observed between cytokine and anti-HPV16 VLP antibody responses.

3.3. Comparison between cytokine responses to L1 VLP in whole blood and isolated PBMC

We and others have previously reported that L1 VLP vaccination is associated with increases in IFN-γ, IL-10, and IL-5 in isolated PBMC using ELISA [8–10]. To compare cytokine profiles in whole blood from vaccine recipients to the ones seen with isolated PBMC, we evaluated the effect of vaccination on ex-vivo cytokine production in response to L1 VLP by PBMC from 6 of the 20 vaccine recipients tested in whole blood assays for those we had available cryopreserved PBMCs (Fig. 2). As observed in whole blood cultures (Fig. 1), stimulation of PBMC from vaccine recipients with L1 VLP (10 μg/ml) induced significant increases in Th1 (IL-2, IFN-γ, TNF-α, GM-CSF) and Th2 (IL-4, IL-5, IL-6, IL-10) type cytokines ($p < 0.05$) (Fig. 2A). In contrast to results from the whole blood assay, no increases were found in IL-1β or IL-8 production to the vaccine in PBMC from vaccine recipients following vaccination. Interestingly, PBMC assays showed increases in IL-5 production in response to L1 VLP, which were not detectable in whole blood assays (Fig. 2A). Similar results were found when L1 VLP was used in vitro at 1.0 μg/ml, although the increases detected following vaccination were much smaller, due to the dose dependent effect of the antigen in cytokine induction.

As observed with whole blood, L1 VLPs had a background stimulating effect on IL-2, IL-6, IL-8, IL-10, and IFN-γ production in PBMC collected at month 0 (before initial immunization), particularly at a concentration of 10 μg/ml (Fig. 2A). Also, no significant increases ($p > 0.05$) in cytokine responses in unstimulated PBMC cultures or cultures stimulated with the positive controls (PHA or Flu) were observed during the course of the study (Fig. 2B and C).

Cultures stimulated with Baculovirus lysate, as control for the production system of the L1 VLPs, showed no statistically

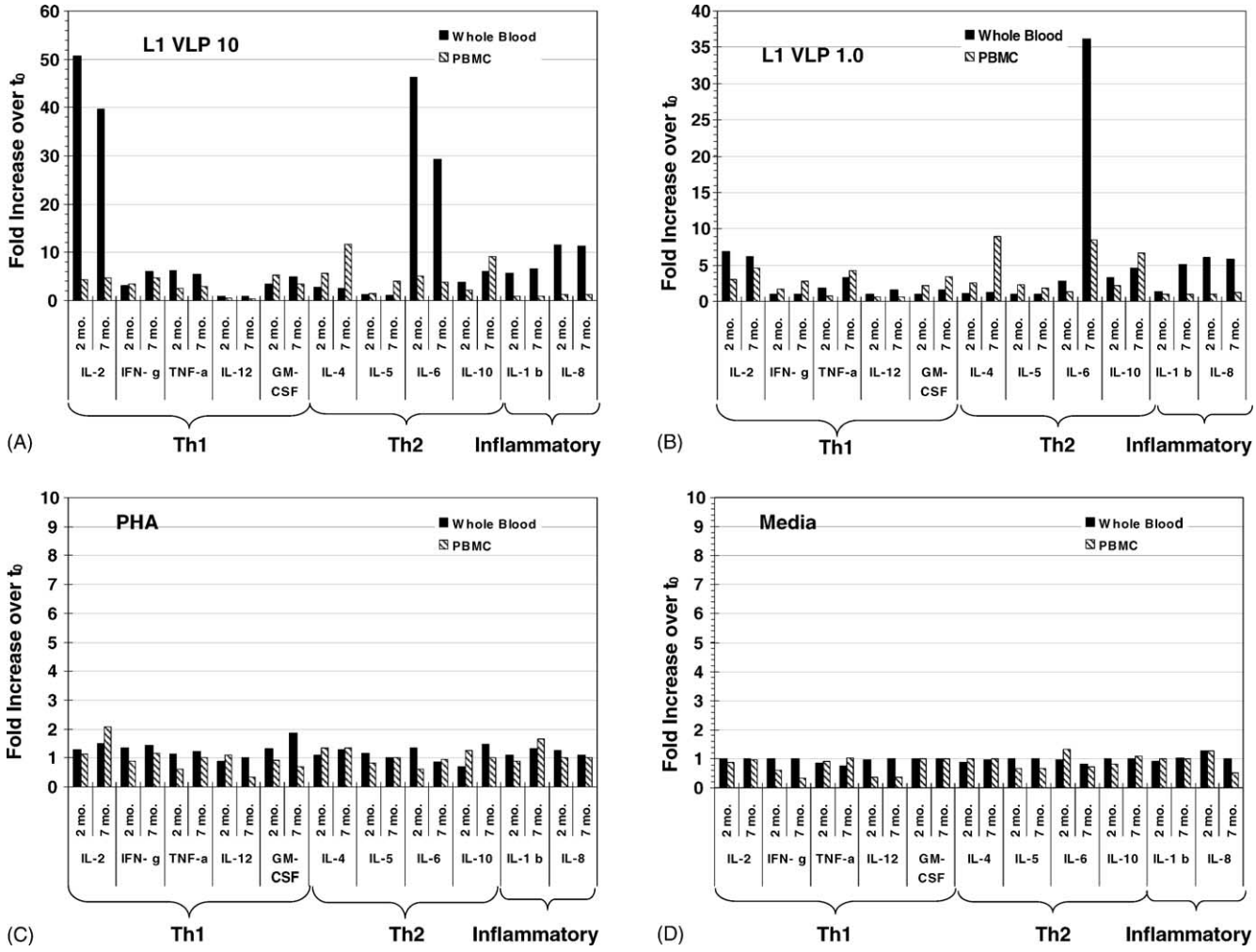

Fig. 3. Comparison between cytokine responses to L1 VLP tested at 10 μg/ml (A) or 1 μg/ml (B), PHA (C) and media control (D) at months 2 and 7 following vaccination in whole blood (filled bars) and matched isolated PBMC (hatched bars). PBMC and whole blood cultures from six vaccine recipients were stimulated as indicated in Section 2. Supernatants were collected and tested using multiplex cytokine kits. Results were expressed as median fold increase levels produced at month 2 or month 7 over the levels produced before vaccination (month 0, t_o).

significant increases after vaccination, except for IL-2 and IL-4 production ($P_{Trend} < 0.05$), but the increases seen for these cytokines tended to be lower than the increases seen in L1 VLP stimulated cultures (Fig. 2A and C). This reactivity may indicate immune responses to antigens of the system of production of L1 VLP vaccine preparations or responses to cross reactive antigens.

A direct comparison between results obtained with PBMC and matched whole blood cultures, expressed in median fold increase over month 0 levels, is shown in Fig. 3. While increases over $t(0)$ were seen for Th1 and Th2 cytokines using both whole blood and PBMC assays, relative increases were higher for PBMC compared to whole blood for IL-4 (5.7- and 11.8-fold versus 2.7- and 2.6-fold, at months 2 and 7, respectively) and IL-5 (1.5-and 4.0-fold versus 1.2-fold at both time points, respectively). Conversely, relative increases were higher for whole blood compared to PBMC for IL-2 (50.7- and 39.8-fold versus 44.5- and 4.8-fold, at months 2 and 7 respectively), IL-6 (46.3 and 29.2-fold versus 5.2 and 3.8-fold, respectively), IL-1β (5.7- and 6.5-fold versus 1.0-fold at both time points, respectively), and IL-8 (11.5- and 11.4-fold versus 1.3- and 1.2-fold at both time points in PBMC and whole blood, respectively).

4. Discussion

In this study, we have demonstrated that a recall response to L1 VLP vaccine results in an overall increase in cytokine production, including adaptive and innate responses. This broad-spectrum increase in cytokine production was seen using an ex-vivo whole blood assay. Whole blood multiplex cytokine assays distinguished clearly between placebo and vaccine recipients and between pre- and post-vaccination samples among vaccinees, demonstrating its use in monitoring vaccine trials. Interestingly, some differences between PBMC and whole blood assays were observed, with higher levels of induction for most cytokines found in whole blood. The lack of correlation seen between the various cytokines and L1 VLP antibodies measured suggest heterogeneity in the immune response to HPV vaccination between individuals, despite the fact that immunization results in the uniformly strong induction of antibody titers [7]. Whether these differences in immune response to vaccination will correlate with duration of protection afforded by HPV vaccination remains to be determined.

This is the first published report to utilize multiplex technology for evaluation of cytokine profiles in a vaccine study using an in vitro whole blood assay. Also, this is the first study to our knowledge to report increases in innate/inflammatory cytokines in the course of mounting a recall response to a prophylactic antiviral vaccine, illustrating the potential cross talk between innate and adaptive arms of the immune system [29,30]. The increased production of inflammatory cytokines in response to L1 VLP is consistent with the activation of antigen-presenting cells, such as monocytes and dendritic cells, or granulocytes by the L1 VLP seen in in vitro assays [26,28]. Other subunit vaccines have been demonstrated to induce inflammatory cytokines. Bordetella pertussis toxin induces release of IL-1, IL-6, TNF-α, [31] and foot and mouth disease vaccine has been reported to activate IL-6 and IL-8 production [32]. In addition, the increase of innate immune responses to L1 VLP in vitro after vaccination may be a result of interactions between activated T cells and antigen-presenting cells or granulocytes through soluble mediators and costimulatory signals such as CD40L and CD40 [33–35]. Although both assays detect increases in Th1 and Th2 type cytokines after vaccination, whole blood assays allowed identification of vaccination-induced increases in cytokines not detectable in PBMC (IL-1β, IL-8) or detectable at lower relative level (IL-2 and IL-6). This was in some cases due to the fact that VLPs induced higher level of cytokines in PBMC than in WB prior to vaccination. The induction of cytokines by VLPs seen prior to vaccination could not be explained by seropositivity at enrolment, since only one of the 20 vaccine recipients were seropositive prior to vaccination. However, this observation does not exclude the possibility that the responses detected at enrolment could be due to previous exposures to HPV. Serology measurements may underestimate exposure to HPV due to low seroconversion and waning seropositivity over time. Alternatively, the responses to VLP seen prior to vaccination could be due to the fact that L1 VLPs are highly immunogenic and can activate production of multiple cytokines in a memory independent fashion [29,30].

The difference in results obtained with whole blood or PBMC might also be explained by the different composition in leukocyte subsets in whole blood versus ficoll-isolated PBMC. Whole blood contains much higher levels of granulocytes (usually higher than 50%) than gradient-isolated mononuclear cells (which usually contain less than 10% granulocytes). Ficoll-isolated PBMC are enriched in lymphocytes when compared to whole blood (approximately >70 versus ≤40%, respectively). Neither the whole blood or the PBMC assays reveal the phenotypic features of the responding cell and the cytokines measured are potentially produced by multiple cell types. Collection of stimulated supernatants in whole blood cultures (24 h cultures) and isolated mononuclear cells (72 h) was done at different time points. Activation of leukocytes in whole blood and isolated PBMC may follow different kinetics [36]. Due to the limited sample availability, supernatants were collected at a single time point that was previously determined to allow detection of most of the cytokines under the different conditions tested.

Increases in Th1 and Th2 type cell cytokines (IL-2, IFN-γ, IL-5, IL-10) have been reported to occur following vaccination with L1 VLP vaccines [8–10]. The increases in the production of T cell cytokines (IFN-γ, IL-2, IL-4, IL-10) probably correspond to the restimulation of memory responses to VLP because only VLP stimulated cultures from blood donors that had been vaccinated exhibited significant increases in those cytokines.

In conclusion, the results presented here indicate that (1) cellular immune responses to HPV-16 L1 VLP vaccination are broad and include up-regulation of both the adaptive and innate arms of the immune system, (2) whole blood assays can clearly distinguish between unvaccinated and vaccinated individuals, (3) differences exist in the response profile observed when whole blood and PBMC are used, and (4) despite the strong antibody induction invariantly observed following vaccination, the underlying cellular immune response varies considerably between individuals.

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